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REMOVAL OF THE FERTILIZATION MEMBRANE OF FERTILIZED EGGS OF *URECHIS CAUPO* AND DEVELOPMENT OF "MEMBRANELESS" EMBRYOS [1]

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Embryos of *Urechis caupo* are invested by a fertilization membrane, and unlike many other invertebrates, embryos of *Urechis* do not lose this membrane during "hatching" of the blastulae. The removal of the fertilization membrane is a prerequisite to embryo dissociation; embryo dissociation has been employed recently to investigate the role of cell interaction in the regulation of biosyntheses (Giudice, Mutulo and Moscona, 1967; Pfohl and Giudice, 1967; Shiokawa and Yamana, 1967; Sconzo, Pirrone, Mutolo and Giudice, 1970; Engstrom, in preparation). This paper describes a method to remove the fertilization membrane and the subsequent development of the "membraneless" embryos.

Materials and Methods

Collection and culture of gametes

Specimens of *Urechis caupo* collected from the mudflats of Bodega Bay, California, were housed in aquaria containing aerated recirculating sea water at 8–12° C. The collection and filtering of eggs has been described (Gould, 1967). Unless otherwise stated, all incubations of eggs and embryos were at 15° C. A one per cent suspension of eggs (vol/vol) was inseminated with a fresh suspension of sperm. Five minutes later, those eggs destined for demembranization were washed and resuspended in millipore-filtered sea water (FSW). Control eggs (with membranes) were similarly washed, resuspended at 2° C in FSW containing 0.1 mg each of penicillin and streptomycin/ml (PSSW) and then transferred to a 15° C water bath for subsequent development. Embryos were cultured in silicone-treated glass petri dishes in volumes not exceeding 5 mm in height in order to allow good aeration and development of the embryos. Untreated glass, agar-coated glass and plastic were not suitable containers for culturing membraneless embryos due to adherence of denuded embryos to these substrates.

Removal of the fertilization membrane

When the fertilization membrane (FM) commenced to rise (10–15 minutes post fertilization), eggs were centrifuged, resuspended in calcium-free sea water (28.4 g NaCl, 5.4 g $MgCl_2 \cdot 6H_2O$, 0.78 g KCl, 7.12 g $MgSO_4 \cdot 7H_2O$, 0.2 g $NaHCO_3$ per liter, pH 7.8—all reagents were analytical grade from Mallinckrodt) containing 1 m*M* disodium ethylenediamine tetraacetate, pH 7.8–7.9 (CFSW-EDTA), centrifuged and finally resuspended at 2° C in 20–25 volumes of a sucrose-EDTA solution (SE)—0.87 *M* sucrose, 5 m*M* EDTA, pH 7.8–7.9. The

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egg suspension was transferred to a 15° C water bath and periodically inverted very gently to mix the suspension. Fertilization membranes became progressively thinner until they were no longer visible by light microscopy; this typically required 10–15 minutes. At the first sign of complete dissolution of the FM, the suspension was gently diluted with 30 volumes of FSW. The above treatments always resulted in the lysis of some eggs (5–20%). The final suspension was centrifuged at 40 × *g* for ½ minute in a Sorvall GSA rotor. These demembranated eggs were resuspended at a concentration of 1% in PSSW (assuming no loss of eggs during SE treatment) and cultured under the same conditions as the controls. When necessary, the cultures were gently rotated to resuspend any embryos adhering to the siliconized glass.

Mechanical treatment, thiol reagents, proteolytic enzymes, nonelectrolytes and combinations thereof were tried without success in removing membranes. Although prolonged exposure to urea or pronase did remove the FM, further development was inhibited. See Berg (1967) for a review of methods of FM removal.

TABLE I

Removal of the fertilization membrane. Fertilized eggs were treated with the reagents listed under Treatment; the duration of exposure is noted under Time; the Results indicate whether the FM remained (+) or was removed (−) following treatment

Treatment	Time (post fertilization)	Results
Sucrose (0.87 M), 15°C	8 minutes–24 hr	+
Sucrose (0.87 M) + EDTA (1 mM), 15° C	7 minutes–1¾ hr	±
Sucrose (0.87 M) + EDTA (10 mM), 15° C	10–20 minutes	− (no division by 7 hr)
Sucrose (0.87 M) + EDTA (5 mM), 2° C	10 minutes–2½ hr	+
Sucrose (0.87 M) + EDTA (5 mM), 15° C	10–20 minutes	− (good development)
Sucrose (p.87 M) + EDTA (5 mM), 15° C—unfertilized eggs in FSW at 2° C for 6 hr. prior to fertilization	10–40 minutes	+

OBSERVATIONS

Removal of the FM

A non-electrolyte (0.87 *M* sucrose) in combination with a chelating reagent (5 m*M* EDTA) proved to be effective both in removing the FM of freshly fertilized *Urechis* eggs (10–25 minutes postfertilization) or blastulae, and in allowing development to ensue. As can be seen from Table I, too high a concentration of EDTA (10 m*M*) led to FM⁻ eggs which did not continue development, while too low a concentration (1 m*M* EDTA) removed the FM of only a small proportion of the eggs. The FM appeared to be solubilized at about the same rate over its entire surface. The temperature of incubation in SE proved to be important, 15° C being effective while 2° C was not. In addition, if unfertilized eggs were held at 2° C for 6 hours, though they fertilized and developed well, their FMs were resistant to dissolution.

Development of embryos lacking FM

Through the 4-cell stage, FM⁻ embryos lagged by 5–10 minutes, thereafter the embryological development of membraneless and control embryos seemed equivalent as far as rate of development and morphology of developmental stages (Figs. 1a and b). Cleavage planes were the same in control and experimental embryos for the period studied, *i.e.,* through the 16-cell stage. FM⁻ embryos were able to form respectable blastulae which began swimming shortly after 9 hours. Culture dishes of demembranated embryos always contained a small number of isolated cells. Gastrulation began at 17 to 17½ hours in control and experimental embryos. Both control and FM⁻ embryos exhibited a concentration of red pigment granules (hematin (Baumberger and Michaelis, 1931)) on the surface around the blastopore. Generally, the diameter of the blastopore of an FM⁻ early gastrula was 1.5 times that of the control. By 21 hours, the embryos were well-defined gastrulae. At this time a small population of green pigment granules was present on the surface below the prototroch over the region of the future intestine. Newby (1940) states that *Urechis* does not possess green pigments though two other species of echiuroids do (*Thalassema* and *Echiurus*). By 25 hours, both experimental and control embryos possessed a tuft of long apical cilia and the appropriate divisions of the gut. At this time, one difference in these embryos had become quite apparent, the membraneless embryos were more elongate (Fig. 1c and d). This difference was more strikingly expressed as development proceeded. At the level of the prototroch in the trochophore larva (43 hours), the width of the experimental embryo was typically only about 0.7 while its longitudinal axis was 1.3 times that of the control embryo (Fig. 1e and f). By two days, FM⁻ and control embryos were feeding and able to contract along their longitudinal axis, notably changing their shapes. Newby (1940) observed such contraction at four days of development. An unexpected difference between FM⁻ and control embryos was that at all swimming stages, the membraneless embryos were less motile than the corresponding control embryos. Unless noted, the embryology described above agrees with that presented in Newby's monograph on *Urechis* development (Newby, 1940).

Lastly, cultures of FM⁻ embryos always contained some double-embryos whereas the controls did not. Sometimes these embryos were merely "joined" at some point, usually at the pretrochal level; more often, their apical ends were "fused," there being a digestive system at each end (Fig. 1g). It may be inferred that one function of the FM is, so to speak, to keep an embryo unto itself. This same phenomenon of double embryos has been observed in the sea urchin (Vacquier and Mazia, 1968b).

From the above observations, it was concluded that removal of the FM does not interfere with normal embryogenesis in terms of such events as blastulation, gastrulation, ciliation or pigmentation, but does have some influence upon the motility, shape and "oneness" of these embryos. Biochemical data suggest that FM⁻ embryos are normal in their time of initiation of synthesis of rRNA (Engstrom, in preparation).

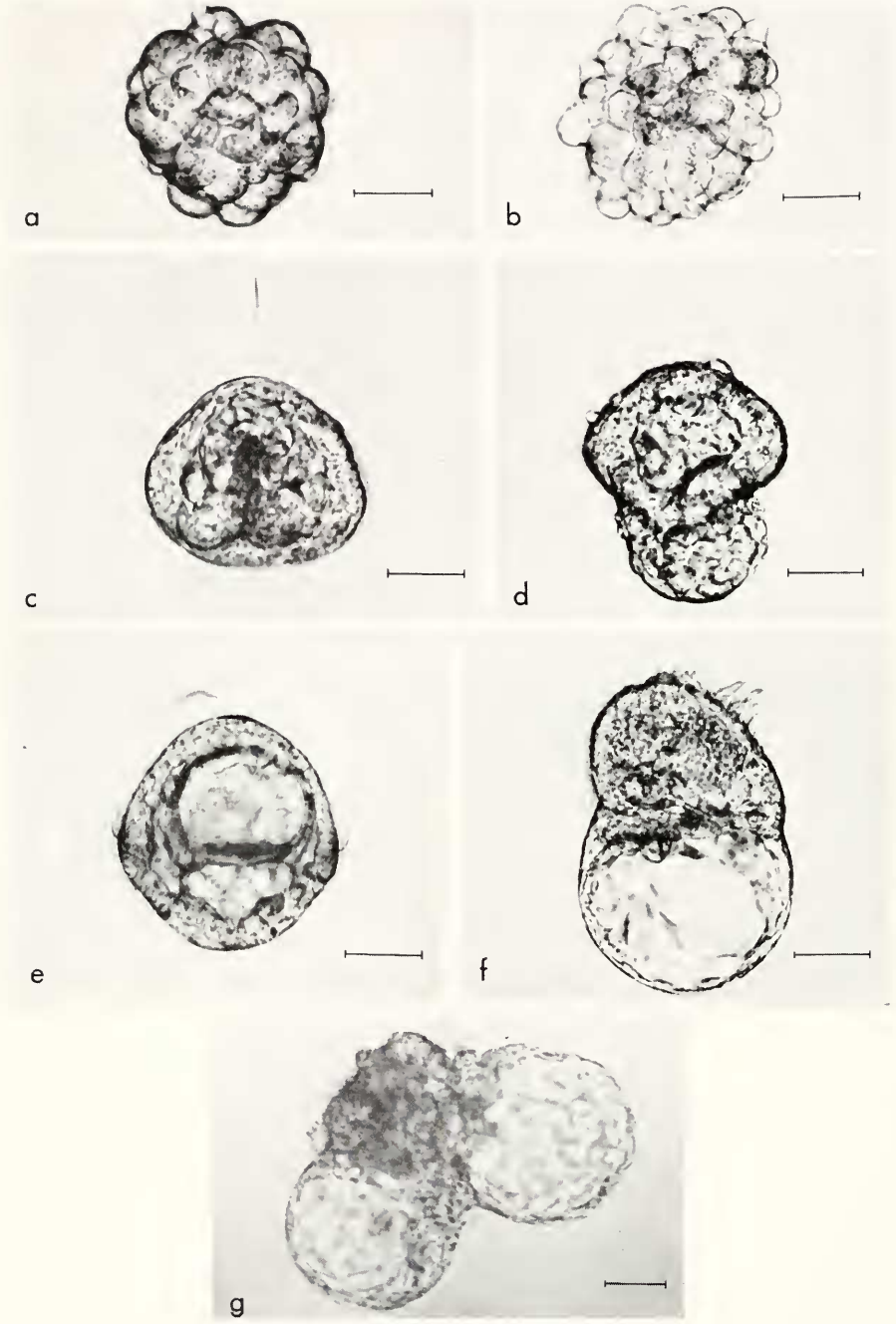

FIGURE 1. *Urechis* embryos as viewed by light microscopy: (a.) 6¾ hour FM$^+$, apical view; (b.) 7½ hour FM$^-$, apical view; (c.) 24 hour FM$^+$, latero-posterior view; (d.) 24 hour FM$^-$, latero-anterior view; (e.) 46 hour FM$^+$, lateral view; (f.) 46 hour FM$^-$, lateral view; (g.) "Double" embryos (FM$^-$) at 4 days as viewed by light microscopy; lateral view. Bar on figure represents 50 μ.

Discussion

Mechanism of membrane removal

In hindsight, it was not surprising that a nonelectrolyte along with a chelating agent would remove the FM from a marine embryo which appears to retain its FM into late development. The necessity to invoke the use of a chelating agent in addition to sucrose suggests that ions, especially divalent cations, play a role in the integrity of the FM of *Urechis* eggs. These findings are in line with Temin's (1956) proposal that the FM of the *Urechis* egg is composed of two layers, an outer protein layer, soluble in nonelectrolyte, and an inner calcium-proteinate layer. In addition, the removal of the FM from unfertilized eggs of *S. purpuratus* with non-electrolyte can be prevented by the addition of ions, particularly divalent cations (Dean and Moore, 1949; Moore, 1932).

The sensitivity of membrane removal to temperature may mean that by 2½ hours EDTA had not yet had access to the Ca-proteinate layer. It is possible, though not to be expected, that Ca^{++} ion deposition in the membrane was greater or the bonding more tenacious at lower temperature. Unfertilized eggs seem to "age" and render the FM more resistant to the action of SE. It is difficult to account for this finding but, since the eggs were not observed for a long period of time, it is possible the FM would have eventually dissolved. Resistance to treatment cannot have been due to the usual "hardening" process since the FM can be removed at least as late as the blastula stage.

The unexpected difference in the degree of motility between the membraneless and control embryos might be explained by supposing that the FM has an energy-providing role. Since the cilia pass through the FM this seems a reasonable hypothesis. A second alternative is that some component(s) of the cilia may have been solubilized or inactivated during exposure of the embryo to SE. It has been shown that some components of isolated cilia of *Tetrahymena* are solubilized in low ionic strength-EDTA buffer (Renaud, Rowe and Gibbons, 1968). Since SE treatment of *Urechis* embryos preceded the appearance of cilia by at least 7 hours, the above speculation presumes: (1) the existence of a pool of ciliary proteins and (2) that the effect of this nonelectrolyte-chelating solution (SE) would be at the level of ciliary protein units or subunits. Evidence for a pool of ciliary proteins has been reported by Auclair and Siegel (1966). Lastly, the present observations cannot exclude the possibility that FM^- embryos simply have fewer cilia and so are less motile than FM^+ embryos.

Development of membraneless embryos

Up through the trochophore stage, the membraneless embryos appeared normal in their embryological development though abnormal in shape. This difference was first detected at the beginning of gastrulation. Recall that the blastopore of the FM^- embryo was larger than that of the normal embryo and that older experimental embryos were longer and narrower than controls. The explanation for the earlier observation is probably that during invagination, the FM being relatively inelastic, acts as a restraint against outward directed cell movements. One would expect this lack of restraint to be reflected in the overall diameter of the blastula, especially in the vegetal region. At about 48 hours of development, the archenteron of the control embryo has undergone distention and stretching. By this time,

cuticle formation has also begun (Newby, 1940). Without the restraining influence of the FM, this distention of the archenteron might cause the membraneless embryo to become more elongate. One can conclude that there was no cuticle formation to prevent this shape change in FM⁻ embryos. The importance of the FM for the shape of the embryos suggests that the FM is retained into later development. Support for this point comes from studies on worms from another phylum, Sipunculoidea. First it should be recalled that unlike sea urchins, *Urechis* blastulae do not shed the FM when they "hatch," rather the cilia pass through the FM. This same behavior is seen in three different genera of sipunculids; further, the FM persists in the sipunculid trochophore and is transformed into the cuticle of the metamorphosing larva (Rice, 1967).

Further, either a hyaline layer is normally absent, removed by nonelectrolyte, or if present, is unimportant in influencing the shape of the embryo. Circumstantial evidence suggests that there is no hyaline layer in FM⁻ embryos. Moore (1930) found that pretreatment of unfertilized *S. purpuratus* eggs with nonelectrolyte prevented the formation of a FM or hyaline layer upon fertilization. A more direct kind of evidence is that FM⁻ *Urechis* embryos were able to form double embryos. This has also been found with sea urchin embryos which are lacking a hyaline layer (Vacquier and Mazia, 1968b). Assuming that no hyaline layer is present in FM⁻ embryos, one can infer that intercellular bonding between blastomeres is important in the maintenance of structure of the embryos. This is similar to the case in the sand dollar, *Dendraster excentricus*, in which cell-hyaline layer interaction is weak with intercellular bonding assuming the dominant role in binding blastomeres together (Vacquier and Mazia, 1968a). In summary, it may be that the FM and intercellular bonding are important in maintaining the shape of an *Urechis* embryo.

Summary

It has been shown that the fertilization membrane of the *Urechis* embryo can be removed by treatment with a sucrose-EDTA solution. The resulting membraneless embryos appear to undergo nearly normal embryogenesis, differing from the controls only in shape, degree of motility and ability to form double-embryos.

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